# Phytoplankton pigments in oceanography

Jeffrey, Mantoura & Wright *eds*., 1997

# Evaluation of methods and solvents for pigment extraction

Wright *et al*., 1997

# Sonication

- Sonication in dimethyl formamide gave the best pigment extraction of all protocols tested and may thus be regarded as the ‘Reference’ extraction method.

- Sonication in methanol appears to be a practical alternative to DMF.

# Soaking

- Soaking without mechanical disruption should not be used as an extraction method since recovery is low, variable, and always accompanied by degradation products.

# Freezing

- Freezing of the sample before extraction did not appear to increase extractability, and the effect on chlorophyllase activity depended markedly on the subsequent extraction technique.

# Acetone

- For spectrophotometric analysis, acetone may still be used to extract pigments from diatoms and naked flagellates, since there are accurate simultaneous equations for Chls *a*, *b*, and *c* in this solvent (Jeffrey & Humphrey, 1975).

# Methanol and DMF

- Porra *et al*. (1989) published equations for Chls *a* and *b* in both methanol and DMF allowing use of these solvents for green algae or other samples where Chl *c* is not present.

# Filtration and storage of pigments from microalgae

Mantoura *et al*., 1997

# Glass-fiber - Advantages

- High filtration capacity and rates.
- Insoluble in pigment-extracting solvents.
- GF/F retains >94% of picoplankton, *Synechococcus* sp., *Prochlorococcus* sp., and picoeukaryotes.
- Aids cell disruption.
- Less expensive than membrane filters.
- Compatible with HPLC, TLC and elemental (C, H, N) analyses.

# Glass-fiber – Disadvantages

- Broad size cut-off.
- Unsuitable for size-fractionation.

# Membrane – Advantages

- Available in wide range of narrow pore sizes (0.01-10 μm).
- Suitable for size-fractionation of phytobiomass and production.
- Compatible with SP, SF, fluorometry and gravimetry.

# Membrane – Disadvantages

- Low filtration capacity and rates.
- Cellulose-ester filters dissolve in pigment-extracting solvents; therefore unsuitable for HPLC, TLC.
- Nucleopore polycarbonate filters release dyes that interfere with HPLC.
- More expensive than glass-fiber filters.

# $MgCO_3$ addition

- **Advantages:**
- Buffers pH, improves particle retention.

- **Disadvantages:**
- Absorbs chlorophyllides and phaeophorbides.
- Retards filtration.

# Recommendations on filters and storage

- Whatman GF/F (0.7 μm) or equivalent filters are recommended for most sampling procedures in which pigments are analyzed by TLC or HPLC.

# *-196ºC (Liquid nitrogen)*

- Storage of filters of phytoplankton under liquid nitrogen (-196ºC) is recommended for the preservation and recovery of pigments from filtered samples for up to 328 days.

# *-90ºC (Ultra-cold freezer)*

- Storage of filters in an ultra-cold freezer (-90ºC) achieves excellent pigment recoveries with minimum degradation for at least 60 days.

# *-20ºC (deep freezer)*

- Long-term storage of phytoplankton filters at -20ºC is not recommended, but if storage is short-term (not more than one week) good pigment recoveries with minimum degradation of Chl *a* may be obtained.

# *Freeze-drying (+22ºC)*

- Freeze-drying causes rapid loss and extensive degradation of Chls and carotenoids in filters of all microalgae tested: about 25% loss in 1 day and at least 80% loss in 328 days.

# *Degradation during storage*

- Whenever Chl *a* was lost during storage, chlorophyllide *a*, Chl *a* allomers and epimers were consistently the most prominent and diagnostic degradation products of inadequate treatment.

# Comparison between spectrophotometric, fluorometric and HPLC methods for chlorophyll analysis

Mantoura *et al*., 1997

# SCOR-UNESCO

- Intercalibration of Lorenzen and Jeffrey (1980) set a benchmark for objective comparisons by using analytically pure pigment standards and mixtures to test the accuracy of several spectrophotometric and fluorometric methods.

# SCOR WG78

- Comparisons between HPLC and one fluorometric and three spectrophotometric methods tested on 11 mixtures of pure chlorophylls and derivatives, 29 microalgal extracts, and 14 seawater and sediment samples.
- HPLC method was first validated against the primary mixture of pure pigments, then used for the spectrophotometric and fluorometric method comparison.

# Conclusions

- Determination of Chl *a* in simple pigment mixtures and extracts free of Chl degradation products is accurately done using the JH spectrophotometric method (<5% error) and the H-H fluorometric method if Chl *b* is absent.

# Conclusions (cont.)

- Acid-spectrophotometric and fluorometric methods for Chl *a* of L and H-H reduce, but do not eliminate, interference from phaeophytins and phaeophorbides.

# Conclusions (cont.)

- Welschmeyer's (1994) optical improvements to the H-H fluorometric method were not tested here, but seem to significantly reduce interference from Chl *b* and pheopigments and increase the fluorometric selectivity for Chl *a*.

# Conclusions (cont.)

- Since the spectra of chlorophyllide *a* and *b* are identical to Chl *a* and *b*, they cannot be distinguished from their parent Chls by either spectrophotometry or fluorometry, unless a phase separation step is included.

# Conclusions (cont.)

- In practice, a limited number of representative samples must first be screened by TLC or HPLC to ensure the absence of interfering degradation products.
- Thus the advantages of pigment spectroscopy in terms of simplicity, accuracy, cost and throughput can be fully realized.
- However, the only methods that can accurately assess all Chls in the presence of degradation products are separation techniques such as TLC and HPLC.

# Guidelines for collection and pigment analysis of field samples

Wright & Mantoura, 1997

# Matching the method to the question

- The concentration of pigments in phytoplankton provides a measure of the phytoplankton biomass.
- This information is valuable for oceanographers, fisheries and water management authorities, and environmental agencies.
- The types of data required varies from an ‘approximate’ measurement of Chl *a* to a full analysis of chlorophylls, carotenoids and degradation products.
- The aim may be to produce surface maps, vertical profiles, time series, grazing or sedimentation profiles, pigment mass balances and so on.
- The choice of method depends on the type of data required, the type and number of samples, and the equipment and time available.

# *In vivo* fluorometry

- Approximate Chl *a* only.
- < 1 min.
- 15 ml.
- Immediate data; sensitive.
- Not quantitative; fluorescence signal depends not only on Chl concentration but also on species composition, time of day, accessory pigments, physiological status, fluorescence quenching.
- Interference from Chl derivatives.

# Extracted fluorometry

- Accurate Chl *a*.
- 10 min.
- 20 ml.
- Very sensitive; accurate if no Chl *b* present; inexpensive.
- Interference from Chl derivatives.

# Spectrofluorometry

- Accurate Chls *a*, *b*, *c*.
- 15 min.
- 50 ml.
- Very sensitive.
- Interference from Chl derivatives; needs continual calibration.

# Spectrophotometry

- Accurate Chls *a*, *b*, *c*.
- 10 min.
- 500 ml.
- Accurate if no Chl derivatives present.

# Thin-layer chromatography

- Chls *a*, *b*, *c*, carotenoids, degradation products.
- 60 min.
- 30,000 ml.
- 2-dimensional TLC gives very good resolution; inexpensive; excellent for pigment purification.
- Not suitable for routine analysis of oceanographic samples.

# Isocratic HPLC

- Accurate Chls *a*, *b*, *c*, some carotenoids, degradation products.
- 25 min.
- 1000 ml.
- Good for major Chls; simpler and faster than gradient HPLC; suitable for shipboard use.
- Medium resolution permits analysis of simple samples only (*e.g.*, cultures or Chls only in field samples).

# Gradient HPLC

- Accurate Chls *a*, *b*, *c*, carotenoids, degradation products.
- 40 min.
- 1000 ml.
- Excellent resolution and quantitation; very sensitive for low Chls and derivatives with fluorescence detection; suitable for shipboard use.
- Expensive to set up; time-consuming for sample preparation, analysis, and data workup (not shown in time estimate).

# Collection of samples

- Opaque bottles, filtered immediately (<1 hr).
- Protect from light and warmth.
- Gently mixed before subsampling.

# *Removal of zooplankton*

- Large zooplankton present significant problems for pigment analysis:
- Their lipids interfere with chromatography,
- A single organism may contain sufficient Chl in its gut to overwhelm the phytoplankton Chl.

# *Filtration*

- A glass-fiber filter such as Whatman GF/F (0.7 μm) should be used for HPLC sampling.
- $MgCO_3$ should not be used as a filter aid since it preferentially absorbs chlorophyllides.

# *Storage of filtered samples*

- Frozen in liquid N, may be kept for several days at –20ºC, or for weeks at –90ºC.

# Fluorometry and spectroscopy of sample extracts

- Although sonication in methanol has been recommended for extraction for HPLC, spectrophotometric equations to correct for absorption by Chls *b* and *c* have not yet been developed for this solvent as they have been for 90% acetone.

# Spectrophotometric and fluorometric equations in common use in oceanography

Jeffrey & Welschmeyer, 1997

# Summary

- Most of our current knowledge of phytoplankton distributions in the ocean is based on Chl analyses made by spectrophotometry and filter-fluorometry.
- Both techniques will continue to be used whenever simple assays for Chl *a* are required.

# Summary (cont.)

- Fluorometry offers significant advantages in sensitivity which result in its popularity in providing simple, low cost Chl *a* analyses in most ocean environments.
- All pigment assays, including fluorometric and HPLC techniques, will ultimately be referenced to spectrophotometric absorbance measurements and, in this regard, spectrophotometric techniques are indispensable.

# Summary (cont.)

- Where concentrations of accessory Chls are needed, the dichromatic and trichromatic spectrophotometric techniques can provide good accuracy for Chls *a* and *b*, *a* and *c*, and *a*, *b*, and $c_1$+$c_2$, provided that sample absorbances are high and degradation products are absent (*e.g.*, algal cultures).

# Summary (cont.)

- If accurate analyses of accessory Chls and degradation products are required, especially on natural field samples, then isocratic HPLC is recommended.
- If the full suite of Chls, carotenoids and Chl degradation products in field samples is required, then an HPLC method should be used.

# Summary (cont.)

- Fluorometric analysis of Chl *a* is accurate under conditions where Chl *b* is absent.

- The accuracy of estimated phaeopigment concentrations is questionable, especially under common conditions where the ratio Chl/phaeopigment is high.

# Summary (cont.)

- *In vivo* fluorometry provides the most convenient, but least accurate method of determining Chl *a*, and has become popular for studies involving long term monitoring and small-scale spatial distributions.
- Currently no substitute for *in vivo* fluorescence for rapidly mapping real-time, vertical and horizontal resolution in phytoplankton biomass over both temporal and spatial dimensions.